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CHEMICAL MODELS FOR THE CHEMICAL NATURE OF ENDOGENOUS DIGITALIS

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The inability or the capacity to promote the phosphorylation of Na^+/K^+-transporting ATPase (Na/K-ATPase) from [^{32}P] P_i is shown to differentiate between mechanistically digitalis-unlike and digitalis-like inhibitors of this enzyme known to be the receptor for all digitalis actions. A negative or positive response in the phosphorylation promotion assay introduced here appears thus to be suitable to diagnose the chemical species in the isolates of animal origin related to the putative endogenous digitalis. Various digitalis-congeneric C/D-cis steroids, progesterone-congeneric C/D-trans steroids and the Erythrophleum alkaloid cassaine promote the enzyme phosphorylation and show a similar pattern of discrimination between three Na/K-ATPase variants. Thus, their cyclopentanoperhydrophenanthrene or perhydrophenanthrene nuclei appear to serve as the minimal pharmacophoric lead structures for bimolecular recognition and to represent chemical models for the chemical nature of endogenous digitalis. Specifically, the hormonal C/D-trans steroids could provide the basic skeleton in endogenous digitalis biosynthesis.

KEY WORDS: Na^+/K^+-transporting ATPase, inhibitor differentiation, enzyme phosphorylation promotion assay, endogenous digitalis, chemical models, minimal structure for bimolecular recognition

INTRODUCTION

Since Szent-Györgyi suggested in 1953 that the digitalis cardiac glycosides are not drugs at all, but are substitutes for a missing screw in the contractile machinery of heart muscle which had a cardinal role in one of the most basic physiological regulations[1], the research on endogenous digitalis has become a challenge to many[2-7]. Due to the use of diverse, apparently unspecific assays[2-7] there has been some confusion in this field as summarized in the following statements[7]: at least 19 isolates have partially been characterized, but all apparently contained different chemical species so no deduction could be made as to the chemical nature of endogenous digitalis; there are no good chemical models for its nature; the field seems to be burdened by an inability to define a unique research strategy. Here, we propose a way to overcome the above hindrances.

The Na^+/K^+-transporting ATPase (EC 3.6.1.37, Na/K-ATPase), present in the plasma membrane of all excitable tissues, has stood the test as the molecular point of attack (receptor) for all digitalis actions[8]. An inhibitory effect on the enzyme or on the Na^+/K^+ pump has generally been accepted as a prerequisite to initially evaluate any material as putatively containing a digitalis-like factor[3-7]. Indeed, numerous substances of non-digitalis structure are known to inhibit Na/K-ATPase[9], but most may

[+] Correspondence.

safely be assumed not to do so by a digitalis-like submolecular mechanism (a few examples are illustrated below). Hence, an inhibitory action on Na/K-ATPase or Na^+/K^+ pump is a necessary but, alone, by no means a sufficient condition to classify an endogenous or exogenous substance as acting digitalis-like.

In our view[10], a digitalis-like acting inhibitor is exactly defined by its property to competently occupy the digitalis recognition cleft in the Na/K-ATPase protein. The recognition cleft is presumably formed by all four extracellularly exposed α-peptide chain segments of the enzyme[11]. The amino acids in the segments are genetically highly conserved[11]. This makes likely not only that an endogenous counterpart to the digitalis compounds of herbal origin exists in the animal body, but also that the three-dimensional and electrostatic structure of the digitalis recognition cleft[10] provides the analytically required tool to pick the digitalis-like acting inhibitors from any set of inhibitory agents of disparate chemical type and to map the complementary structure of the interfaces involved with respect to the chemical nature of endogenous digitalis. This has been the basic working hypothesis underlying our present search into chemical models for the chemical nature of endogenous digitalis.

The competent occupancy of the digitalis recognition cleft by any inhibitor (I) of Na/K-ATPase (E) and hence its nature as a mechanistically digitalis-like compound appears to be proved by the promotion of enzyme phosphorylation from orthophosphate (P_i):

$$E \underset{P_i \cdot Mg^{2+}}{\overset{(1)}{\rightleftharpoons}} \overset{Mg}{E} \cdot P_i \overset{(2)}{\rightleftharpoons} \overset{Mg}{E} \sim P \overset{(3)}{\underset{I}{\rightleftharpoons}} I \cdot \overset{Mg}{E} \sim P \underset{I + P_i . Mg^{2+}}{\overset{(4)}{\rightleftharpoons}} E$$

The progress of the reaction sequence is thermodynamically highly demanding. After negentropy-producing binding of Mg^{2+} and P_i to the enzyme[12], Na/K-ATPase synthesizes, entropically driven[13], the phosphoenzyme $E \sim P$ with an aspartyl phosphate residue in the catalytic centre[14]. The envelopment of a digitalis-like acting inhibitor by the peptide chain segments of the digitalis recognition cleft leads to a large entropy gain in the inhibitor-phosphoenzyme complex[15,16] which expresses itself in a stabilization of the phosphoenzyme once formed. The outcome is a shift in equilibrium (2) to the right thus elevating the actual phosphoenzyme level measured[14]. A negative or positive response in this phosphorylation promotion test introduced in the present study allows the discrimination between compounds which inhibit Na/K-ATPase activity through a digitalis-unlike mechanism as they do not promote, or even suppress, enzyme phosphorylation (**1** to **3** in Table I), and those which do so through a digitalis-like mechanism as they promote enzyme phosphorylation in a concentration- and structure-dependent degree (**4** to **21**).

METHODS AND MATERIALS

The preparation of the three Na/K-ATPase variants, the determination of their enzymatic activity by the coupled optical assay and the assessment of its inhibition by the compounds enumerated in Tables I and II were done as described[17,18]. The enzyme phosphorylation from [^{32}P] P_i was performed as described earlier[19]. The source of the inhibitors were indicated[17-20]. Compound **8** was a gift by Dr. LaBella[21].

RESULTS AND DISCUSSION

Progesterone-bisguanylhydrazone (**1**; cf. Figure 1) was reported to be no longer hormonally active, but to show digitalis-like actions on heart muscle[22]. The recent suggestion that it operates through binding of the guanylhydrazone functions near the binding subsites in the digitalis recognition cleft for lactone and sugar side chains of cardiac glycosides[23], is disfavoured by the finding that **1**, contrary to the digitalis representative **5**, does not promote enzyme phosphorylation (Table I). Cibacron Blue (**2**), a sulfonated polyaromatic dye, suppresses the phosphorylation (Table I) by occupancy of the catalytic centre[24]. The antiestrogen tamoxifen (**3**) does not influence the phosphorylation reaction, despite its geometric similarity to estradiol-17β[25]. However, the antiestrogen megestrol acetate (**4**) which has, like the digitalis compounds, a steroid nucleus, although not with C/D-cis but C/D-trans geometry, does promote enzyme phosphorylation. This surprising observation will be evaluated below in connection with the properties of other C/D-trans pregnane derivatives (**10** to **19**).

The probe with various C/D-cis steroids (**5** to **9**; cf. Figure 1), shows that the absence of the lactone function at C-17β (in **6** to **9**), the absence of the sugar function at C-3β (in **6**) and the absence of the hydroxyl function at C-14β (in **9**) do not eliminate the capacity to promote enzyme phosphorylation, but only change the concentration required to reveal it (Table I). In conclusion, all digitalis-characteristic substituents on the steroid nucleus, which are typically absent in the hormonal C/D-trans steroids, are not required for competent occupancy of the digitalis recognition cleft.

The major remaining difference between digitalis and hormonal steroids is then the

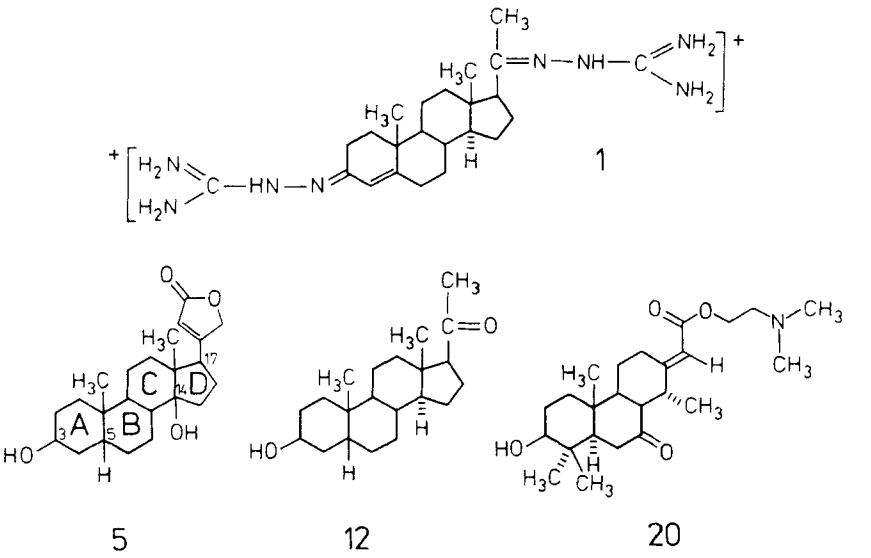

FIGURE 1 Structural formulae of progesterone-bisguanyl-hydrazone (**1**), digitoxigenin (**5**), 3β-hydroxy-5β-pregnan-20-one (**12**) and cassaine (**20**). In **1**, the guanylhydrazone functions dominate the digitalis-unlike mechanism of Na/K-ATPase inhibition. In **20**, however, the dimethylaminoethyl side chain plays a reinforcing role only. Compounds **5** and **12** typify a digitalis-like C/D-cis steroid and a progesterone-like C/D-trans steroid, respectively. The common minimum lead structure for bimolecular recognition between Na/K-ATPase and mechanistically digitalis-like inhibitors exemplified by **5**, **12** and **20** appears to be the perhydrophenanthrene moiety.

TABLE I

Effect of various compounds on Na/K-ATPase activity from human brain cortex and enzyme phosphorylation from [^{32}P]P_i

Compound	Inhibitor, systematic (trivial) name	Concentration (μM)	Inhibition (%)	Phosphoenzyme (%)
–	No inhibitor (control)	–	–	26 ± 7
1	Pregn-4-ene-3,20-bis-guanylhydrazone (progesterone-bisguanylhydrazone)	100	100	8 ± 8
2	Cibacron Blue F3GA	200	93	0 ± 0
3	(E)-1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1,2-diphenyl-but-1-ene (tamoxifen)	20	98	29 ± 2
4	17α-Acetoxy-6-methyl-pregna-4,6-diene-3,20-dione (megestrol acetate)	10	39	64 ± 13
5	17β-(But-2′-en-4′-olid-3′-yl)-5β, 14β-androstane-3β, 14-diol (digitoxigenin)	100	100	100 ± 0
6	5β, 14β-Androstane-3β, 14-diol	200	49	48 ± 2
7	3β-Rhamnosyloxy-5β, 14β-androstan-14-ol	30	71	75 ± 6
8	3β-Rhamnosyloxy-5β, 14β-pregnane-14,20β-diol	100	96	103 ± 8
9	14-Amino-3β-rhamnosyloxy-5β, 14β-pregnan-20β-ol	3	99	100 ± 3
10	17α-Acetoxy-6-chloro-3β-hydroxy-pregna-4,6-dien-20-one (chlormadinol acetate)	10	58	46 ± 6
11	3β-O-Rhamnosyl-chlormadinol acetate	10	16	24 ± 2
13	3β-Rhamnosyloxy-5β-pregnan-20-one	20	74	33 ± 3
15	17α-Acetoxy-6α-methyl-3β-rhamnosyloxy-5β-pregnan-20-one	80	100	79 ± 7
16	17α-Acetoxy-3β-hydroxy-pregn-5-en-20-one	10	6	32 ± 9
17	17α-Acetoxy-3β-rhamnosyloxy-pregn-5-en-20-one	10	35	44 ± 6
19	17α-Acetoxy-6-methyl-3β-rhamnosyloxy-pregn-5-en-20-one	50	81	61 ± 6
20	Cassaic acid dimethylaminoethyl ester (cassaine)	3	62	49 ± 3
		30	97	72 ± 5
		300	100	91 ± 2
21	Cassaic acid methyl ester	200	50	38 ± 4

The maximum phosphorylation amounted to 1003 fmol per enzyme unit, defined as the activity splitting one μmol ATP per hour. Phosphoenzyme values are the means of at least two independent triple determinations, supplemented by the average deviations from the means. The inhibitors **12**, **14** and **18** specified in Table II, could not adequately be characterized in the phosphorylation promotion assay because of limited solubility. The lack of a direct correlation between the efficacy in inhibiting enzyme activity and promoting enzyme phosphorylation is caused by the different ligation of the proper enzyme states involved in the two assays. Generally, the divergence is the greater the lower the $-\Delta G^{01}$ value is (cf. Table II).

cis- and trans-junction of the rings C and D, respectively. This geometric distinction seemed to be decisive, since the C/D-trans gestagen chlormadinol acetate (**10**), although being a potent Na/K-ATPase inhibitor, produced a negative inotropic action[20,21], whereas the C/D-cis pregnane derivative (**8**) elicited positive inotropy[21]. However, annihilation of the hormonal property of chlormadinol acetate by glycosidation[10] revealed sustained positive-inotropic efficacy[20]. These findings eliminate the last barrier for envisaging that C/D-trans steroid derivatives, produced in the animal metabolic pathways, could provide the basic skeleton in endogenous digitalis biosynthesis.

Summing up, the message derivable from the findings with the steroids **5** to **9**, **4** and **10** to **19** shown in Table I simply appears to be that the cyclopentanoperhydrophenanthrene nucleus is their common pharmacophoric lead structure.

The negative or positive impact of 3β-*O*-rhamnosylation on the potency of the C/D-trans steroids **10** to **19** to promote enzyme phosphorylation is a function of the angle of the A/B ring junction. Steroids with unsaturations in rings A and (or) B are known for their conformational flexibility. At least in the recognition cleft of Na/K-ATPase, chlormadinol acetate (**10**) appears to adapt a planar conformation as in A/B-trans steroids, whereas the pregnenes **16** and **18** appear to adapt a bent conformation as in the A/B-cis steroids **12** and **14**. This inference emerges from the findings[26] that the rhamnosides of chlormadinol acetate **10** and of 3β-hydroxy-5α-pregnan-20-one show similarly lowered $-\Delta G^{01}$ values, whereas the rhamnosides of 17α-acetoxy-3β-hydroxy-pregn-5-en-20-one (**16**), 17α-acetoxy-3β-hydroxy-6-methyl-pregn-5-en-20-one (**18**), 3β-hydroxy-5β-pregnan-20-one (**12**) and 17α-acetoxy-3β-hydroxy-6α-methyl-5β-pregnan-20-one (**14**) develop similarly increased $-\Delta G^{01}$ values (cf. Table II).

The Erythrophleum alkaloid cassaine (**20**; Figure 1) shares many of the pharmacological actions of the digitalis steroids[27], but seems to satisfy none of the structural requirements usually associated with their properties. Surprisingly, cassaine (**20**) and also cassaic acid methyl ester (**21**) are able to promote enzyme phosphorylation and may thus be classified as mechanistically digitalis-like acting compounds. The dimethylaminoethyl side chain of cassaine may cause its local-anaesthetic effect[28], whereas the perhydrophenanthrene nucleus may act as the pharmacophoric lead structure.

In addition to the positive/negative decision about the inhibitory mechanism obtained by the phosphorylation promotion test (Table I), the determination of the apparent Gibbs energy change in the interaction between inhibitors of various type and Na/K-ATPase from different tissues and species might offer a finer distinction between mechanistically digitalis-like inhibitors of variant structure as illustrated in Table II. The digitalis-congeneric C/D-cis steroids **5** to **8** show a typical discrimination pattern characterized by highest $-\Delta G^{01}$ values with the human cardiac enzyme, somewhat smaller values with the human cerebral enzyme and much lower values with the guinea-pig heart muscle enzyme. Remarkably, a quite similar interaction energy pattern is for the most part found with the C/D-trans steroids **4** and **10** to **19** as well as cassaine (**20**). This underlines the above conclusion that these structurally digitalis-non-congeneric inhibitors nonetheless do operate through a digitalis-like submolecular mechanism, i.e., competent occupancy of the digitalis recognition cleft. This clearly does not apply to other types of inhibitors such as **1** to **3** in Tables I and II.

Examination of Table II suggests that 3β-O-rhamnosylation of C/D-trans steroids

TABLE II

Apparent Gibbs energy change in the interaction between inhibitors of various type and Na/K-ATPase preparations from human brain cortex (a), human cardiac muscle (b) and guinea pig cardiac muscle (c)

Compound	Inhibitor, systematic (trivial) name	$-\Delta G^{01}$ (kJ/mol)		
		a	b	c
1	Pregn-4-ene-3,20-bis-guanylhydrazone (progesterone-bisguanylhydrazone)	34.0	33.4	36.5
2	Cibacron Blue F3GA	28.5	26.9	28.6
3	(E)-1-[4-[2-(Dimethylamino)ethoxy]phenyl]-1,2-diphenyl-but-1-ene (tamoxifen)	31.7	30.1	n.d.
4	17α-Acetoxy-6-methyl-pregna-4,6-diene-3,20-dione (megestrol acetate)	28.5	30.4	23.8
5	17β-(But-2′-en-4′-olid-3′-yl)-5β,14β-androstane-3β,14-diol (digitoxigenin)	42.0	43.4	34.0
6	5β, 14β-Androstane-3β,14-diol	21,8	22.7	18.7
7	3β-Rhamnosyloxy-5β, 14β-androstan-14-ol	29.2	30.8	21.0
8	3β-Rhamnosyloxy-5β, 14β-pregnane-14,20β-diol	34.3	n.d.	n.d.
9	14-Amino-3β-rhamnosyloxy-5β,14β-pregnan-20β-ol	45.1	45.2	37.4
10	17α-Acetoxy-6-chloro-3β-hydroxy-pregna-4,6-dien-20-one (chlormadinol acetate)	30.5	32.0	22.5
11	3β-O-Rhamnosyl-chlormadinol acetate	25.5	26.6	26.2
12	3β-Hydroxy-5β-pregnan-20-one	23.8	n.d.	n.d.
13	3β-Rhamnosyloxy-5β-pregnan-20-one	30.6	n.d.	n.d.
14	17α-Acetoxy-3β-hydroxy-6α-methyl-5β-pregnan-20-one	27.1	n.d.	n.d.
15	17α-Acetoxy-6α-methyl-3β-rhamnosyloxy-5β-pregnan-20-one	33.1	n.d.	n.d.
16	17α-Acetoxy-3β-hydroxy-pregn-5-en-20-one	22.6	24.5	n.d.
17	17α-Acetoxy-3β-rhamnosyloxy-pregn-5-en-20-one	28.0	29.9	23.8
18	17α-Acetoxy-3β-hydroxy-6-methyl-pregn-5-en-20-one	23.2	24.2	n.d.
19	17α-Acetoxy-6-methyl-3β-rhamnosyloxy-pregn-5-en-20-one	28.5	31.4	23.8
20	Cassaic acid dimethylaminoethyl ester (cassaine)	34.8	36.7	34.4
21	Cassaic acid methyl ester	22.0	n.d.	n.d.

For quantitative comparison of the inhibitory efficacies, the apparent dissociation constant of the inhibitor-enzyme complex, K_D', was converted into the apparent Gibbs energy change, $-\Delta G^{01}$, by means of the equation $\Delta G^{01} = RT \cdot \ln K_D'$ as founded elsewhere[17,18,29]. n.d. = not determined. The neural and contractile tissues of vertebral animals are known to contain three or two isoforms of Na/K-ATPase that have not yet been isolated[30]. So, the enzyme preparations from human brain cortex and human cardiac muscle probably consisted of isozyme mixtures of unknown different composition. This excluded any simple explanation for the difference in the affinity of the enzyme preparations from human neural and contractile tissues to the various digitalis-like acting C/D-cis and C/D-trans steroids **5** to **9** or **4** and **10** to **19**, respectively.

with a bent A/B ring junction positions the rhamnosyl side chain so as to nicely fit the sugar enveloping segment of the digitalis recognition cleft. The interaction energy increment is namely with the rhamnosylation of **12** and **18** to give **13** or **19**, respectively, as great as that with the rhamnosylation of ouabagenin to give ouabain (−7.0 kJ/mol)[17]. Strikingly, the $-\Delta G^{01}$ values are, for the rhamnosides of the pregnanes **13** and **15** as well as for the pregnenes **17** and **19**, as large as the value for the rhamnoside of the digitalis lead structure **7** (for definition see[17]). This appears to mean that the digitalis recognition cleft envelops equally well C/D-trans and C/D-cis steroid glycosides despite their shape differences, in what appears to indicate a close "induced fit". As already documented above by the negative impact of 3β-O-rhamnosylation on the potency of chlormadinol acetate (**10** → **11**), the cleft has no infinite conformational adaptability. For instance, in digitoxigenin (**5**), the conversion of C/D-cis into C/D-trans configuration annuls the inhibitory activity[17], apparently because this positions

the bulky lactone ring so that it conflicts with neighbouring peptide chain segments of the cleft.

Our results have several implications. The phosphorylation promotion assay is suitable to pick from various compounds or "factors" the mechanistically digitalis-like inhibitors of Na/K-ATPase; the assay may thus provide an essential tool in the research on endogenous digitalis. The cyclopentanoperhydrophenanthrene nucleus in cardiac C/D-cis steroids and hormonal C/D-trans steroids, and the perhydrophenanthrene nucleus in the alkaloid cassaine emerge as the minimal pharmacophoric structures for bimolecular recognition and hence as fitting models for the chemical class of endogenous digitalis. Through competent occupancy, they elicit closing of the digitalis recognition cleft that appears to operate as a switching device[17] in producing the peptide chain rearrangement which underlies the stabilization of the phosphoenzyme intermediate and the inhibition of the enzyme activity. In conjunction with the known primary structure of the peptide chain segments involved in ouabain binding[11], our results presented here and in an earlier paper[10] invite the design of an operational model for the digitalis recognition cleft by means of graphics computer-aided receptor mapping[31,32]. This should allow the generation of ideas for the methodical derivatization of the above pharmacophoric lead structures to approach further the proper chemical structure of endogenous digitalis, and, it is to be hoped, to design novel cardiotonic drugs following the argumentation of Szent-Györgyi[1] referred to in the introduction.

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